METHODS OF SCREENING AND USING INHIBITORS OF ANGIOGENESIS

This patent application is a continuation of co-pending application Serial No. 10/115,718 filed on April 3, 2002 which, in turn, claimed the benefit of U.S. Provisional Application Serial No. 60/281,512, filed April 4, 2001, which is hereby incorporated by reference herein.

Background of the Invention

Angiogenesis is the method by which new blood vessels form from existing vasculature in an animal. The process is distinct from vasculogenesis, in that the new endothelial cells lining the vessel arise from proliferation of existing cells, rather than differentiating from stem cells. The process is invasive and dependent upon proteolyisis of the extracellular matrix (ECM), migration of new endothelial cells, and synthesis of new matrix components. Angiogenesis occurs during embryogenic development of the circulatory system; however, in adult humans, angiogenesis only occurs as a response to a pathological condition (except during the reproductive cycle in women).

Thus, in adults, angiogenesis is associated with conditions including wound healing, arthritis, tumor growth and metastasis, as well as in ocular conditions such as retinopathies, macular degeneration and corneal ulceration and trauma. In each case the progression of angiogenesis is similar: a stimulus results in the formation of a migrating column of endothelial cells. Proteolytic activity is focused at the advancing tip of this "vascular sprout", which breaks down the ECM sufficiently to permit the column of cells to infiltrate and migrate. Behind the advancing front, the endothelial cells differentiate and begin to adhere to each other, thus forming a

new basement membrane. The cells then cease proliferation and finally define a lumen for the new arteriole or capillary.

Due to the fact that certain pathologies including many cancers, retinopathies, arthritis, and macular degeneration depend upon angiogenesis, it would obviously be desirable to find methods for inhibiting angiogenesis associated with these conditions. Preferably such methods would not inhibit the angiogenesis involved in wound healing and other beneficial responses to angiogenic stimuli.

The matrix metalloproteases (MMPS) are a family of proteases that specifically degrade portions of the EMC. These secreted and membrane-associated extracellular proteins are widely considered to be involved in angiogenesis, probably being responsible, at least in part, for creating the opening in the ECM through which the growing vascular sprout can extend during angiogenesis. However, the specific molecular targets of the MMPs are the subject of some debate, as are the mechanisms by which the MMPs may influence other endothelial cell functions such as attachment to the ECM, detachment and migration.

Most MMPs are secreted as zymogens, which are activated in the ECM. The exception is MT1-MMP, which is bound to the cell surface and processed within the cell before migration to the cell membrane. A family of inhibitors of MMPs termed TIMPs (tissue inhibitors of metalloproteases) are antiangiogenic, but, having multiple and complex effects on the angiogenic process, they appear to possess activities in addition of those of a simple competitive inhibitor.

Formation of a vessel during angiogenesis requires the tight adhesion of neighboring endothelial cells in the basement membrane; this adhesion is mediated by members of the integrin superfamily. These transmembrane proteins consist of heterodimers comprising α and β subunits. There are various subtypes of each of the α and β subunits; thus α subunits may include α_3, α_4, α_5, α_6, α_7, α_8, α_9, α_{2b}, α_E

and α_V, while the β subunits may include β_1, β_3, β_5, and β_6. As indicated in further detail below, there is specificity in most cases as to which α subtype can pair with which β subtype. Many, but not all, of the alpha subunits are expressed as an inactive pro form that is then cleaved by a protease termed convertase. Dimerization of these covertase-susceptible subunits appears to require convertase cleavage.

Endothelial cells express integrins in response to various factors including vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ) and basic fibroblast growth factor (bFGF). The expressed integrins mediate cell migration, proliferation, survival, and regulation of matrix degradation.

It has been reported that metalloprotease MT1-MMP, in conjunction with integrin $\alpha_V\beta_3$, activates MMP-2 in cultured breast carcinoma cells by converting the latter from a pro-form to the active form of the enzyme. This activation is inhibited by the introduction of vitronectin, a specific ligand of $\alpha_V\beta_3$. Deryugina E.I., et al., *Exp Cell Res.* 15;263(2):209-23 (Feb. 2001). Additionally, it has been reported that MT1-MMP is capable of activating $\alpha_V\beta_3$ by cleavage of the β_3 subunit when breast cells are transfected with MT1-MMP and theβ_3 subunit. Deryugina E.I., et al., *Int. J. Cancer* 86(1):15-23 (April 2000). Both of these references are incorporated by reference herein.

Summary of the Invention

The present invention is related to the discovery that the matrix metalloprotease MT-1-MMP is capable of activating certain integrins by cleavage of the α subunit. We have discovered that this metalloprotease modifies the α_V subunit of integrin $\alpha_V\beta_3$, the integrin widely thought to be associated with VEGF-mediated angiogenesis. Additionally, MT1-MMP is capable of activating, or

increasing the activation state of, any α subunit that is susceptible to cleavage by convertase. Such subunits include α_3, α_4, α_5, α_6, α_7, α_8, α_9, α_{2b}, α_E and α_V. The MT1-MMP substrate may be the inactive pro-form of the α chain or may be the convertase-cleaved active form. In the latter case, MT1-MMP results in an increase in the activation state of the already active subunit.

Thus, MT1-MMP appears to be part of an angiogenic activation cascade involving integrin heterodimers. Such integrins may include, without limitation, $\alpha_V\beta_3$, $\alpha_V\beta_1$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, and $\alpha_5\beta_1$. As activation of integrin is a prerequisite for initiation of the angiogenic response, means of inhibiting such activation would be a valuable and useful therapeutic tool in the treatment of pathological conditions in which angiogenesis is at least partly a causative or perpetuating factor.

Thus, in one embodiment the invention relates to methods for screening agents which inhibit an angiogenic response comprising contacting together an inactive or convertase-activated integrin α subunit, an agent to be tested for the ability to inhibit angiogenesis, and metalloprotease MT1-MMP under conditions promoting the modification of the integrin α subunit in the absence of said agent, and correlating inhibition of an increase in α subunit activation with the ability of the agent to inhibit angiogenesis. In preferred embodiments, the MT1-MMP and pro form of the integrin α subunit are expressed within the same cell. Also, in a preferred embodiment, the correlating step is accomplished by observing a difference in migration of the MT1-MMP activated form versus the inactive form of the alpha subunit in electrophoresis or chromatography, as the former forms appear to migrate at a different molecular weight.

In another embodiment, the invention relates to a method of treating a patient suffering from a pathological condition in which angiogenesis is at least partially a causative or perpetuating factor with an agent capable of inhibiting an increase of a pro form or convertase-activated form of the integrin α subunit by

MT1-MMP metalloprotease. In preferred embodiments, the pathological condition is selected from the group selected from arthritis, tumor growth, metastasis, retinopathies, macular degeneration, retinal neovascularization, corneal ulceration and corneal trauma.

In this embodiment of the invention, the agent may be administered by any means effective to direct the agent to the affected site. For example, without limitation, in the case of treatment of a tumor, the agent may be injected directly into tumor tissue, preferably into the periphery of the tumor mass; in the case or arthritis, the agent may be injected into the joint; in the case of ocular conditions the agent may be applied via an intraocular implant, such as a bioerodable or reservoir-based drug delivery system for direct treatment of the retina or cornea, or may be formulated in a ophthalmologically acceptable excipient and directly injected into the anterior or posterior segment of the eye.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a gel electrophoretogram of nucleic acid resulting from RT-PCR amplification of mRNA present in naiive corneas (lane 1), and 72 hours and 288 hours post cautery corneas (lanes 2 and 3 respectively. Oligonucleotide primers used corresponded to the labels in each row, and are shown in Table 1.

Figures 2 A, 2C, 2E and 2G are photomicrograms of corneal tissue sections frozen 72 hours post-cauterization and immunostained with Factor VIII, fibronectin, laminin and tenacin-C, respectively.

Figure 2 B is a photomicrogram of a corneal tissue section frozen 72 hours post-cauterization and co-immunostained with Factor VIII and collagen type IV.

Figure 2 D is a photomicrogram of a corneal tissue section frozen 72 hours post-cauterization and immunostained with collagen type IV and fibronectin EDA.

Figure 2F is a photomicrogram of a corneal tissue section frozen 72 hours post-cauterization and co-immunostained with collagen type IV and laminin.

Figure 2H is a photomicrogram of a corneal tissue section frozen 72 hours post-cauterization and co-immunostained with collagen type IV and tenascin-C.

Figure 3A, 3C, 3E, and 3G are photomicrograms of tissue sections of the limbal region of naïve corneas immunostained for the α_1, α_2, α_5 and β_5 integrin subunits, respectively.

Figure 3B, 3D, 3F, and 3H are photomicrograms of central corneal region of naïve corneas immunostained for the α_1, α_2, α_5 and β_5 integrin subunits, respectively.

Figures 4 A, 4E and 4I are photomicrograms of corneal tissue samples frozen 72 hours post-cautery and immunostained for α_1, α_2 and β5 integrin subunits, respectively.

Figures 4 C, 4G and 4K are photomicrograms of corneal tissue samples frozen 120 hours post-cautery and immunostained for α_1, α_2 and β5 integrin subunits, respectively.

Figures 4B, 4F and 4J are photomicrograms of corneal tissue samples frozen 72 hours post-cautery and co-immunostained for a) collagen type IV, and b) α_1, α_2, and β_5 integrin subunits, respectively.

Figures 4D, 4H and 4L are photomicrograms of corneal tissue samples frozen 120 hours post-cautery and co-immunostained for a) collagen type IV, and b) α_1, α_2, and β_5 integrin subunits, respectively.

Figure 5A is a photomicrogram of corneal tissue samples frozen 72 hours post-cautery and immunostained for the α_5 integrin subunit.

Figure 5B is a photomicrogram of corneal tissue samples frozen 72 hours post-cautery and immunostained for collagen type IV and the α_5 integrin subunit.

Figure 5C is a photomicrogram of corneal tissue samples frozen 120 hours post-cautery and immunostained for the α_5 integrin subunit.

Figure 5D is a photomicrogram of corneal tissue samples frozen 120 hours post-cautery and immunostained for collagen type IV and the α_5 integrin subunit.

Figure 5E is a photomicrogram of corneal tissue samples frozen 168 hours post-cautery and immunostained for the α_5 integrin subunit.

Figure 5F is a photomicrogram of corneal tissue samples frozen 168 hours post-cautery and immunostained for collagen type IV and the α_5 integrin subunit.

Figure 5G is a photomicrogram of corneal tissue samples frozen 72 hours post-cautery and immunostained for the integrin B_3 subunit.

Figure 5H is a photomicrogram of corneal tissue samples frozen 72 hours post-cautery and immunostained for collagen type IV and the integrin B_3 subunit.

Figure 5I is a photomicrogram of corneal tissue samples frozen 120 hours post-cautery and immunostained for the integrin B_3 subunit.

Figure 5J is a photomicrogram of corneal tissue samples frozen 120 hours post-cautery and immunostained for collagen type IV and integrin B_3 subunit.

Figure 6A is a confocal photomicrogram of whole mounted corneal tissue immunostained for lectin and integrin B_3 subunit in an alkaline burn model; wherein angiogenesis was induced by bFGF in the cornea.

Figure 6B is a confocal photomicrogram of whole mounted corneal tissue samples immunostained for lectin and integrin B_3 subunit in an alkaline burn model, wherein angiogenesis was induced by bFGF in the cornea.

Figure 6C is a confocal photomicrogram of whole mounted corneal tissue samples immunostained for lectin, wherein angiogenesis was induced by bFGF in the cornea. (L) is the limbus and (P) is the location of the pellet containing bFGF.

Figure 6D is a confocal photomicrogram of whole mounted corneal tissue samples immunostained for integrin B_3 subunit, wherein angiogenesis was induced by bFGF in the cornea. (L) is the limbus and (P) is the location of the pellet containing bFGF.

Figure 6E is a confocal photomicrogram of whole mounted corneal tissue samples immunostained for integrin B_3 subunit, wherein angiogenesis was induced by bFGF in the cornea.

Figure 6F is a confocal photomicrogram of whole mounted corneal tissue samples immunostained for lectin and integrin B_3 subunit, wherein angiogenesis was induced by bFGF in the cornea.

Figure 7A is a graphical representation of sections taken through naive and injured corneas.

Figure 7B shows photographs of gelatin zymography from corneas taken from naïve corneas and corneas taken 24, 72, 120, and 168 hours post injury.

Figure 8A-E shows the results of in situ gelatin zymography in naïve corneas and those injured 24 hours, 72 hours, 120 hours, and 168 hours post-injury, respectively.

Figure 9A-D are immunohistograms of frozen corneal sections frozen 72 hours post-injury. Figures 9A is stained form MMP-2 and Figure 9C is stained for MT1-MMP. Figures 9B and 9D are stained for lectin, as well as MMP-2 and MT1-MMP, respectively.

The following examples do not limit the generality of the invention disclosed herein.

Examples

Methods. Neovascularization in female sprague-dawley rats was induced by alkaline cauterization of the central cornea. Corneas from naïve, 72 hrs and 288 hrs post cautery animals were analyzed by RT-PCR for integrins α_1, α_2, β_3, β_5, the endothelial marker CD31, and metalloproteinases MMP-2 and MT1-MMP. Analysis of protein expression and metalloproteinases were conducted in corneas

from naïve, 24, 72, 120, and 168 hrs post cautery animals by immunofluorescent microscopy in frozen sections and gelatin zymography.

Results. RT-PCR indicated a correlation between expression of CD31, MT1-MMP and integrins α_1 and β_3, with neovascularization of the cornea. Immunohistochemical analysis indicated that at the protein level integrins α_1, α_2, α_5 and β_5, and MT1-MMP were expressed on newly developing vasculature while β_3 integrin was expressed at low levels within the neovascular lumen. As previously seen ECM proteins laminin, collagen type IV and fibronectin were expressed throughout the developing vasculature, however, tenascin-C showed preferential staining of maturing vasculature with little or no expression within the invasive angiogenic front. Expression of MMP-9 correlated with corneal epithelial cell migration while MMP-2 expression was associated with inflammatory cell invasion and neovessel formation.

Conclusions. Integrin expression during neovascularization of rat corneas in response to alkaline injury is restricted to angiogenesis along the VEGF/$\alpha_v\beta_5$ pathway in conjunction with $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_5\beta_1$ integrins. Expression of MT1-MMP within the invasive angiogenic front further suggest that MT1-MMP is also important in mediating VEGF driven angiogenic response, potentially in conjunction with $\alpha_v\beta_5$ or β_1 integrins which co-distribute with MT1-MMP. The pattern of Integrin expression observed within this study correlates well with a VEGF mediated angiogenic response.

Angiogenesis within adult tissues is a response to a diverse set of stimuli including angiogenic and inflammatory cytokines that induce a quiescent vasculature to reenter the cell cycle and invade the surrounding stroma producing a new region of vascularized tissue. Central to this process are the activities of both cell adhesion receptors and matrix degrading enzymes belonging to the family of matrix metalloproteinases (MMPs). Inhibition or disruption of either cell adhesion

or MMP activity through genetic manipulations or pharmaceutical intervention is capable of inhibiting an angiogenic response. In many instances the adhesion receptors involved and or MMPs are likely to be dictated by the angiogenic factors present. While this factor dependence has not been well characterized for MMPs, cell adhesion through integrins has been characterized to occur through at least two principle adhesion pathways corresponding to angiogenic induction by either bFGF or VEGF. Thus, in bFGF induced response, which also includes induction by TNF-α, angiogenesis occurs in an $\alpha_v\beta_3$ mediated pathway, induction of angiogenesis by VEGF, as well as TGF-β and PMA, occurs through $\alpha_v\beta_5$. While these two pathways are well established, recent studies suggest that under pathological conditions the correlation between growth factors and integrin expression are not always maintained. In several instances where VEGF is present both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are expressed and in at least one study the functional significance of $\alpha_v\beta_3$ mediated angiogenesis may reflect the presence of ligand for $\alpha_v\beta_3$. Additionally, not all aspects of angiogenesis are dependent on expression of $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins. Knockout mice for α_v as well as β_3 integrin appear to under go extensive vasculogenesis and angiogenesis in the absence of either α_v or $\alpha_v\beta_3$ integrins, although subtle vascular defects are present with both embryonic and post natal lethality observed in association with abnormal vessel formation. These later observations suggest that other integrin family members are capable of complementing the functions of α_v or β_3 integrins or that other adhesive pathways, independent of α_v or β_3 integrins, are present. Other members of the integrin family implicated in mediating an angiogenic response include $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_5\beta_1$ integrins which like α_v integrins have also been divided into bFGF associated ($\alpha_5\beta_1$) or VEGF associated ($\alpha_1\beta_1$, $\alpha_2\beta_1$) angiogenic events. The above studies suggest that within a given angiogenic response the adhesion mediated pathway is likely to be diverse and depend not only on the presence of a single angiogenic

factor but the collective influence of ECM and associated factors including MMPs and inflammatory cytokines.

Recently, the corneal alkaline burn model of angiogenesis has been characterized as having high levels of VEGF present during active vessel growth, suggesting that VEGF is the primary angiogenic factor within this model system. Consistent with this finding, pharmaceutical intervention with $\alpha_v\beta_3$ antagonists has no effect on the angiogenic response, suggesting that angiogenesis occurs through an $\alpha_v\beta_5$ adhesion pathway which is consistent with a VEGF mediated angiogenic response. However, expression of $\alpha_v\beta_5$ was neither established in these studies nor other potential adhesion receptors identified. The purpose of this study was to characterize the pattern of integrin expression to determine if this angiogenic response occurs through a $\alpha_v\beta_5$ mediated pathway as well as characterize other members of the integrin family which may also be functionally relevant to a VEGF mediated angiogenic response. This study addresses these issues by examining both the spatial and temporal expression patterns of integrins relative to the expression of extracellular matrix molecules associated with a neovascular response including collagen type IV, laminin, fibronectin and tenascin-C. Additionally, we have examined the expression of metalloproteinases MMP-2 and MT1-MMP to determine if they are also involved in mediating the angiogenic response.

In conclusion, collagen type IV, laminin and fibronectin EDA domain expression was consistent with previous studies on neovascularization. Tenascin-C, however, showed a unique pattern of expression correlating with vessel maturation. In agreement with a VEGF mediated angiogenic response neovascularization was associated with expression of $\alpha_v\beta_5$, $\alpha_1\beta_1$, and $\alpha_2\beta_1$ integrins as well as $\alpha_5\beta_1$. MMP-2 and MT1-MMP were both associated with the robust inflammatory response as

well as vessel formation. The localization of MT1-MMP to the developing vasculature in the absence of $\alpha_v\beta_3$ suggests that MMP-2 as well as MT1-MMP may have broader roles in mediating an angiogeneic response than previously recognized by their association with $\alpha_v\beta_3$ integrins.

Materials and Methods:

Reagents and antibodies: Brdu (5-bromo-2-deoxyuridine) was purchased from Boehringer Mannheim. TRIzol reagent and SuperScript II reverse transcriptase were from Gibco-BRL (Rockville, MD). Gelatin zymography gels (10% PAGE), renaturing buffer and developing buffer were from Novex (San Diego). Primary antibodies were purchased from the following companies and used at the following concentrations: goat anti-type IV collagen was from Southern Biotechnology Associates, Inc. (Birmingham, AL) and used at 1:250 dilution (1.6 ug/ml); Mouse anti-fibronectin EDA domain, FN-3E2 was from sigma (St. Louis, MO) and used at 1:300 dilution, rabbit anti-human factor VIII was from Dako Corporation (Carpinteria, CA) and used at 1:100 dilution Anti-tenascin-C polyclonal antibody HXB1005 was a generous gift from : Sharifi B.G., and was used at 1:100 dilution; rabbit polyclonal anti-integrin α_1 subunit, -integrin α_2 subunit, -integrin α_3 subunit, -integrin α_5 subunit, -integrin β_5 subunit were from Chemicon International Inc.(Temecula, CA) and used at 1: 100 dilutions for the α subunits and 1:500 dilution for β_5 subunit; mouse monoclonal anti-rat integrin β_3 chain was from PharMingen (San Diego, CA) and used at 1:100 dilution (5 ug/ml); rabbit polyclonal anti-MMP-2, and MT-MMP1 were from Chemicon International Inc. (Temecula, CA) All secondary antibodies were F(ab')2 fragments conjugated to either rhodamine (TRITC) or fluorescein (FITC). They were purchased from

Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and used at 1:200 dilutions.

Animal model. Female rats (Sprague-Dawley), weighing 250-300 gm, were anesthetized with isoflurane (4% v/v) and topical application to the corneal surface with proparacaine 0.1 % Allergan Inc. (Irvine, CA). The alkaline burn is created by touching the central cornea with the tip of a silver nitrate applicator (75% silver nitrate, 25% Potassium nitrate) Grafco™ Graham-Field Inc, (Hauppauge, NY) for 2 seconds. At the indicated times animals were euthanized and the eyes were enucleated at post injury intervals ranging from 24 hrs to 288 hrs for various studies. For immunofluorescence analysis, the eyes were embedded in OCT solution and cryosectioned. For wholemount studies, entire corneas were removed and quartered. Experimental animals were treated and maintained in accordance with ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Cryosectioning and Immunofluorescence. The eyes (injured or naïve) were sagittally cryosectioned in 8 –13 μm sections for immunostaining with mouse monoclonal or goat and rabbit polyclonal antibodies. The sections were fixed in 100% acetone for 5 minutes, briefly dried, rehydrated in phosphate-buffered saline (PBS) and incubated in a moist chamber as follows: 5% BSA (Sigma) in PBS for 2hr, primary antibodies for 2 hr at room temperature, five washes in PBS for 5 min each, secondary antibodies conjugated to fluorochromes for 1 hr at room temperature, five washes as before. Samples were mounted with Fluoromount G (Southern Biotechnology Associates) and observed and photographed with a Nikon E800 compound microscope equipped with a Spot Digital Camera (Diagnostic Instruments Inc. Sterling Heights, MI). Co-localization of the angiogenesis-related molecules and vascular markers were achieved by using various combinations of mouse, goat or rabbit primary antibodies. Negative controls for immunostaining

were the use of naive serum or purified IgG for each species of primary used as well as secondary alone. In all instances tissues were co-stained with Collagen type IV to mark the presence of vessels as well as serve as an internal positive control. All control tissues were from corneas 72 hrs post injury since this provided the greatest range of cellularity.

Whole Mount Immunofluorescence: Complete fresh corneas were cut in quarters and fixed in 90% methanol and 10% DMSO for 15 min at room temperature, rinsed in PBS (1x) 2 min x 3 times, blocked in 2% BSA in PBS for 4 hrs, incubated in primary antibody α2/CD31 or β5/CD31, β3/ Banderaea Simplicifolia (BS-1) lectin overnight at 4°C, washed in PBS 1 hr x 5 times, followed by incubation in second antibodies conjugated to fluorochromes for overnight at 4°C and washed for 1 hr x 5 times. Finally corneas were flat mounted and analyzed by either a Nikon E800 compound microscope equipped with a Spot Digital Camera (Diagnostic Instruments Inc. Sterling Heights, MI) or by Confocal microscopy using a Lecia TCS SP confocal microscope (Leica Microsystems Inc., Exton, PA).

In Situ Zymography: Frozen tissue sections, 4-8 um in thickness were mounted onto gelatin coated slides (Fuji, Pharmaceuticals Inc.) and incubated at 37°C in a moist chamber for 4 hrs to 6 hrs followed by drying at room temperature. After fixation, tissues were stained with Amido Black 10B solution for 15 minutes followed by rinsing in water and then destain (70% methanol, 10% acetic acid) for 20 minutes. Images were captured by bright field microscopy.

RT-PCR: The total RNA was isolated from the pooled corneal tissue (total of four corneas) from naïve, 72 hr and 288 hrs post cautery animals using a standard TRIzol extraction procedure as outlined in the manufacturer's protocol GibcoBRL (Rockville, MD). Isolated RNA was treated with Rnase free DNase I to remove any contaminating genomic DNA. RT-PCR analysis of RNA in the absence of

reverse transcriptase was used as a negative control. The total RNA was quantitated by spectrophotometry at an absorbence of 260 nm. Total RNA (1μg) was reverse transcribed with 50 units SuperScript II reverse transcriptase in the presence of 2.5ug/ml random hexamer and 500 μM dNTP for 50 min at 42 °C, followed at 70 °C for 15 min. 1ul of the resulting cDNA was amplified in the presence of 1nM sense and antisense primers, 200 uM dNTP, and 3.5 units of Expand™ High Fidelity enzyme mix . PCR conditions: Initial 5 cycles, denature at 94 °C for 15 sec, annealing at 58 - 55 °C for 30 sec (decrease 0.5 °C each cycle), and 72 °C for 30 seconds. For the remaining 27 cycles PCR conditions were 94 °C for 15 sec, 55 °C for 30 sec, and 72 °C for 45 seconds. The amplified samples were then loaded at equal volumes (10 μl) onto 1.5% agarose gels. The PCR products were visualized with ethidium bromide. The primer pairs used for amplification are given in Table 1. All PCR products were subcloned and sequenced to verify product as the target gene.

Corneal Micropocket Assay: Corneal Micropocket assay was carried out as described in (23) using 400 ng bFGF / hydron pellet bead. Briefly, Female rats (Sprague-Dawley), weighing 250-300 gm, were put under general anesthetized with 200 μl of (xylazine 20 mg/ml, Ketamine 100 mg/ml and acepromazine) and prior to surgery eyes were topically anesthetized with 0.5% proparacaine. A 1 mm in length corneal incision penetrating half through corneal stroma was made 2.5 mm from the temporal limbus and a pocket was made by separating stroma from the point of incision to about 1mm from limbal vessel. A hydron bead 0.4 x 0.4 mm containing 140 ng bFGF was then implanted in the pocket. Three and five days after implantation of hydron pellet corneas were prepared for whole mount analysis.

Table 1. Oligonucleotide Primer Sequences

Primer	Oligonucleotide Sequence	Fragment size (bp)
MT1-MMP:	5'-GTGACAGGCAAGGCCGATTCG-3'	446
	SEQ. ID NO. 1	
	5'TTGGACAGTCCAGGGCTCAGC-3'	
	SEQ. ID NO. 2	
MMP-2,	5'-ACTCCTGGCACATGCCTTTGCC-3'	401
	SEQ ID NO. 3	
	:5'-TAATCCTCGGTGGTGCCACACC-3'	
	SEQ. ID NO. 4	
integrin β3	5'-TTTGCTAGTGTTTACCACGGATGCCAACAC-3'	866
	SEQ. ID NO. 5	
	5'-CCTTTGTAGCGGACGCAGGAGAAGTCAT-3'	
	SEQ ID NO. 6	
integrin β5	5'-CGAATGGCTGTGAA GGTGAGATTGA-3'	854
	SEQ ID NO. 7	
	5'-CAGTGGTTCCAGGTATCAGGGCTGTAAAAT-3';	
	SEQ ID NO. 8	
integrin α2	5'-CAAGCCTTCAGTGAGAGCCAAGAAACAAAC-3'	728
	SEQ ID NO. 9	
	5'- CAAACC TGCAGTCAATAGCCAACAGGAAAA- 3'	
	SEQ ID NO. 10	
integrin α1	5'-GGAGAACAGAATTGGTTCCTACT TTGG-3'	335
	SEQ ID NO. 11	
	5'-CGGAGCTCCWATCACGAYGTCATTAAATCC-3'	
	SEQ ID NO. 12	
CD31	5'-GGCATCGGCAAAGTGGTCAAG-3'	680
	SEQ ID NO. 13	
	CAAGGCGGCAATGACCACTCC	
	SEQ ID NO. 14	
Actin	5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3'	837

SEQ ID NO. 15
5'-CGTCATACTCCTGC TTGCTGATCCACATCTGC-3'
SEQ ID NO.16

W= A or T, Y=C or T.

Results

To examine the presence or absence of individual integrins and MMPs, RT-PCR was performed examining integrins α_1, α_2, β_3, β_5 and metalloproteinases MMP-2 and MT1-MMP using naïve, 72 hrs (3 days) and 288 hrs (12 day) post cautery corneas. This allowed examination of tissues representing the early (72 hrs) and late phases (288 hrs) of the angiogenic response. Correlation between gene expression relative to vessel growth was accomplished by examining the expression of CD31. Analysis of naïve cornea indicated the absence of messages for CD31, α_1, β_3, and MT1-MMP. Message for MMP-2, β_5, and α_2 integrin was present in naïve corneas (Figure 1). Within injured cornea at both early and late phases of neovascularization α_1, β_3, MT1-MMP, and CD31 mRNA were detected. The correlation between α_1,β_3, MT1-MMP with CD31 expression suggests involvement of the encoded proteins with the neovascular response. Expression of MMP-2, β_5 integrin, and α_2 integrin messages showed no clear change in expression with neovascularization. The absence of a correlation between MMP-2, β_5, and α_2 mRNA with the angiogenic response does not exclude their potential involvement within the angiogenic response but is likely to reflect the limitation of the approach and the relatively high levels already present in naïve corneas. To further refine the analysis, protein expression was examined by immunohistochemical analysis in conjunction with gelatinase zymography. To map the expression of integrins and MMPs to the developing vasculature corneal tissue sections were initially stained for factor VIII to identify endothelial cells as well as a number of extracellular matrix proteins associated with a neovascular

response including collagen type IV, laminin, fibronectin EDA domain and tenascin-C.

Staining in frozen tissue sections from corneas 72 hrs post injury with Factor VIII, collagen type IV, fibronectin EDA domain, laminin and tenascin-C are presented in Figure 2. The entire vasculature as well as distal regions of the developing vasculature were positive for factor VIII, co-immunostaining with collagen type IV showed a similar pattern of vessel staining as that seen with factor VIII, however, collagen type IV did not stain the more distal regions recognized by factor VIII immunostaining (Figure 2B, arrow head), indicating the invasive front proceeds pronounced collagen type IV expression but is factor VIII positive. Coinciding with collagen type IV staining was staining for fibronectin EDA and laminin (Figures 2C-2F). The one exception to the staining observed between collagen type IV, laminin and fibronectin EDA domain was the absence of fibronectin EDA domain staining in the limbal or pre-existing vasculature (Figures 2C and 2D, asterisk). Tenascin-C expression (Figures 2G and 2H), while present within the limbal vasculature, was initially expressed proximal to the initial expression seen for collagen type IV in which a region of collagen type IV positive and tenascin-C negative could be recognized in the more distal regions of vessel formation (Figure 2H, between arrow heads). The rather high levels of tenascin-C seen in the stroma represent remnants of tenascin-C from the scaleral spurr which is rapidly degraded during the initial 24 hrs after corneal cauterization. This later response is restricted to the cautery burn injury as a simple corneal debriment had no effect on the degradation of tenascin within the scaleral spur (data not shown). The staining pattern of ECM is consistent with that which as been previously reported for collagen type IV, fibronectin EDA and laminin, however, the localization of tenascin-C to more proximal regions of the developing vasculature has not been previously reported. The unique staining pattern of tenascin-C relative to collagen

type IV allows identification of a unique region, which may represent a pre-maturation phase in vessel development. Based on the pattern and relative fluorescence intensity, collagen type IV was used to mark the developing vasculature in the following studies examining both integrin and MMP expression.

For the analysis of integrin expression immunological reagents were selected to identify a given integrin pairing. The heterodimer pairs examined in the current study are $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$. Identification of the respective heterodimers was accomplished by staining tissues for α_1, α_2, α_5, β_3, and β_5 integrin subunits. In most cases this allowed the identification of a discrete heterodimer pair since α_1, α_2, and α_5 only pair with β_1 integrin subunit and β_5 only pairs with α_v subunit. The only exception being the anti-β3 antibody which recognizes both the $\alpha_v\beta_3$ and $\alpha_{iib}\beta_3$ heterodimer pairs. However, $\alpha_{iib}\beta_3$ is only expressed on platelets and megakaryocytes allowing elimination based on cell morphology and tissue distribution. Corneas were examined from three separate time courses for each integrin in which cornea staining was examined in naïve, 24, 72, 120 and 168 hrs post cautery. Shown for each of the staining patterns are the 72 and 120 hr time points as these represent the spectrum of staining observed throughout the time course and are believed to represent both early and mid phases of the angiogenic response. Staining in naïve corneas for each of the integrins examined is shown in Figure 3. The majority of staining was seen for α_1, α_2, α_5 and β_5 within the corneal epithelium. Stromal staining was also observed but to a limited extent and not readily apparent (Figure 3). No immunoreactivity was seen for β_3 integrin (not shown).

Staining patterns for α_1, α_2 and β_5 are shown for both the72 hrs. and 120 hrs in the time points in Figure 4. Examination of α_1, α_2, and β_5 at 72 hrs. post injury

indicated similar patterns of expression with staining in the limbal vessels and throughout the developing vasculature co-localizing with collagen type IV immunostaining. Staining of cells within the stroma for α_1, α_2, and β_5 not directly associated with the neovessels was also observed (Figure 4). This latter staining pattern is likely to represent the expression on stromal fibroblast or inflammatory cells which are highly abundant within the stroma at this time point. Expression of α_1 within the developing vasculature showed a uniform pattern of staining throughout the developing vasculature while that for α_2 was variable and punctate. At the120 hrs. time point α_2 showed diminished staining within the leading vascular front (Figures 4G and 4H, asterisk) with pronounced staining within the vasculature frequently observed (Figure 4G, arrow). This latter staining may reflect α_2 expression on platelets or inflammatory cells present within the neovessels. β_5 integrin staining in the developing vasculature was similar to α_1, with expression throughout the developing vasculature (Figures 4I-4L). At the 120 hrs. β_5 continued to show staining throughout the developing vasculature (Figures 4K and 4L). However, preferential staining in more distal regions of the developing vasculature was frequently observed.

Staining for α_5 integrin subunits identifies the presence of the $\alpha_5\beta_1$ heterodimer since α_5 is only known to pair with the β_1 integrin subunit. This integrin heterodimer pair is expressed in multiple cell types and consistent with this pattern of expression α_5 is observed in corneal epithelial and endothelial as well as stromal cells in naïve and injured cornea. Similar to α_1, α_5 staining was uniform throughout the developing vasculature at the 72 hrs. time point (Figures 5A and 5B). At the 120 hrs. time point, α_5 showed localized staining in the more distal regions of the neovasculature (Figures 5C and 5D) and by 168 hrs this differential staining pattern was more pronounced (Figures 5E and 5F). These results from the α_1, α_2, α_5 and β_5

staining suggest within the more distal regions involved in vessel outgrowth, adhesion occurs through $\alpha_1\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_5$ integrins. The Pattern of α_2 staining suggests its potential involvement in the early phases of the angiogenic response but by 120 hrs it is preferentially expressed in regions associated with vessel maturation and remodeling.

Staining for β_3 integrin subunits identifies the presence of either the $\alpha_v\beta_3$ or $\alpha_{iib}\beta_3$ heterodimers. Within naïve cornea β_3 immunostaining is absent (not shown). At 72 and 120 hrs. post injury faint β_3 staining was observed throughout the developing vasculature punctuated by regions of pronounced β_3 immunofluorescence (Figures 5G-5J). Confocal microscopy of whole mounted corneal tissues indicates that the pronounced β_3 immunostaining is associated with expression of β_3 on platelets (Figures 6A and 6B). To confirm that the staining pattern for β_3 is not associated with neovascularization we examined the expression of β_3 in which corneal angiogenesis was induced by bFGF using the corneal micropocket assay. Examination of the bFGF induced neovascularization indicates that β_3 expression is restricted to the leading vasculature front (Figures 6C and 6D) as well as pronounced expression on endothelial cells (Figures 6E and 6F). These results are consistent with previous studies examining $\alpha_v\beta_3$ expression in neovascularized tissue and contrasts greatly with the observed β_3 staining seen in the corneal alkaline burn model. These data indicate that β_3 is not expressed in a fashion consistent with its involvement in mediating endothelial cell adhesion to the extracellular matrix and that the observed β_3 expression is principally expressed on platelets as $\alpha_{iib}\beta_3$.

In addition to the expression of integrins identified by the RT-PCR analysis, message for MT1-MMP was also detected and the presence of this message

correlated with neovascularization of the cornea. Since MT1-MMP is tightly associated with activation of MMP-2 [18,19] we initially examined potential involvement of MT1-MMP by examining the presence of the pro and activated forms of MMP-2 by gelatin zymography. Gelatin zymography was performed on corneas from naïve, 24 hrs, 72 hrs, 120 hrs and 168 hrs post injury. To correlate MMP expression with vessel growth corneas were sectioned as shown in Figure 7A. This provided a relative reference of MMP activity to new vessel growth. In naïve corneas only the pro-form of MMP-2 was present (Figure 7B). At 24 hrs post injury, active forms of MMP-2 were detected in all sections with highest levels present within limbal and wound domains (Figure 7B, sections 1 and 4). At 72 hrs. active forms of MMP-2 were more prevalent in the limbal and adjacent domains forming a gradient with highest levels in the limbal regions (Figure 7B, Sections 1 and 2), suggesting a correlation between the presence of active forms of MMP-2 and neovessel formation. At 120 hrs. the gradient of active forms of MMP-2 extended into the central cornea and by 168 hrs. the gradient had reversed with highest levels seen in the central cornea (Figure 7B, section 4). These data suggest a correlation between vessel growth and MMP-2 activation implicating an active role of MT1-MMP in the angiogenic process.

In addition to MMP-2, MMP-9 expression and activity were also observed by gelatinase zymography. Within 24 hrs post injury pro and active forms of MMP-9 were detected though out the cornea with higher levels seen in sections 3 and 4, representing the wound and adjacent tissue. By 72 and 120 hrs. MMP-9 levels were greatly decreased with only the pro-form detected within the regions of the original corneal wound. This pattern of MMP-9 expression is consistent with expression of MMP-9 during corneal epithelial cell migration.

The complex pattern of MMP-2 activation observed is likely to reflect both active enzyme and that associated with TIMPS as an inactive complex. Additionally,

MMP-2 activity is also like to be associated with inflammatory or stromal fibroblasts not directly associated with the angiogenic process. To identify endogenously active MMP-2 within the cornea in situ zymography was performed (Figure 8). Consistent with the gelatinase zymography the pattern of gelatinase activity as determined by in situ zymography were very similar. In naïve tissue no gelatinase activity was observed and by 24 hrs. a small increase in gelatinase activity was seen through out the cornea. At 72 hrs. gelatinase activity was present within the limbal (Figure 8C, arrowhead) and adjacent regions (Figure 8C, arrow) reflecting the gradient of active forms of MMP-2 observed in the gelatinase zymography. The extent of gelatinase activity extending into the corneal stroma correlates with neovessel formation as previously determined by collagen type IV immunostaining. Additionally, pronounced gelatinase activity was observed within individual cells within the stroma (Figure 8C, asterisk). At 120 hrs. gelatinase activity was similar to that observed at 72 hrs. with the regions of stromal associated gelatinase activity extending further into the corneal stroma correlating with vessel development (Figure 8D). At 168 hrs post injury the majority of gelatinase activity was restricted to individual cells within the central cornea adjacent to the wound. The relatively low levels of gelatinase activity between the limbus and central wound observed in the in situ zymography at 168 hrs. relative to the levels of active forms of MMP-2 observed by gelatin zymography (Figure 7, 168 hrs. time point) suggests that gelatinase activity between the limbus and central cornea are tightly regulated by endogenous TIMPS, consistent with down regulation of MMP activity within regions of vessel maturation.[22] Finally, in the in situ zymography little or no gelatinase activity was seen in relationship to the cornea epithelial cells, this may reflect the inability to obtain adequate development time to allow visualization of an MMP-9 signal. Longer development times often resulted in loss of resolution in the gelatinase activity.

To further define the localization of MMP-2 and expression of MT1-MMP immunohistochemical staining was preformed on frozen corneal sections. Analysis indicated pronounced MPP-2 expression in individual cells within the stroma similar to that seen by in situ zymography with low levels of staining seen in association with developing vasculature (Figures 9A and 9B). MT1-MMP expression was similar to that seen with MMP-2 although higher levels were observed in association with the developing vasculature (Figures 9C and 9D). The strong staining of individual cells within the stroma for MMP-2 suggests that the gelatinase activity seen in the in situ zymography reflects active MMP-2. The gelatinase activity in association with vessel growth may reflect gelatinase activity associated with MMP-2 as well as MT1-MMP, which showed pronounced staining on the developing vasculature correlating with in situ zymography.

Discussion

We examined the pattern of integrin and MMP expression within the corneal alkaline burn model relative to the angiogenic response by RT-PCR, immunofluorescence and gelatin zymography. Initial analysis of integrin and metalloproteinase expression by RT-PCR demonstrated that CD31, integrins α_1 and β_3, and MT1-MMP were expressed in injured cornea correlating with the angiogenic response seen within this model. Expression of α_2, β_5 and MMP-2 indicated no alteration in their pattern of expression relative to neovascularization. The inability to detect a change in message for α_2 integrin, β_5 integrin, and MMP-2 likely reflects the existence of abundant message present in naïve tissues. The expression of MMP-2, β_5 and α_2 in naïve tissue likely reflects the expression of these genes within the corneal epithelium for β_5 and α_2 integrins or within the corneal stroma for MMP-2.

Having identified potential adhesion and metalloproteinases associated with the angiogenic response by RT-PCR we next examined their expression in relationship to vessel formation by immunohistochemical analysis. This was accomplished by initially examining vessel development using the endothelial cell marker factor VIII as well as a number of ECM proteins associated with neovessel development, this included collagen type IV, fibronectin EDA domain, tenascin-C and laminin. From this analysis collagen type IV, fibronectin EDA domain, and laminin stained the entire developing neovasculature with the exception of the more distal regions which were only positively stained for factor VIII. The absence of a clear basement membrane staining at the more distal regions of the developing neovessels is consistent with the observations of Paku and Paweletz, 1991 in which a defined

basement membrane is absent within the invasive tips of vascular buds. The Pattern of collagen type IV, laminin and fibronectin expression is similar to that reported by others examining basement membrane formation during angiogenesis in adult tissue, although, we did not see preferential expression of laminin preceding collagen type IV as reported by Form et al., 1986 during alkaline burn induced corneal neovascularization in the mouse. Both collagen type IV and laminin as well as factor VIII stained the preexisting limbal vasculature while no staining for fibronectin EDA domain was seen. This is consistent with embryonic forms of fibronectin only being expressed in newly developing vasculature in adult tissues or within large vessels. Proximal to the initial staining by collagen type IV was staining of tenascin-C which extend throughout the developing vasculature and into the pre-existing limbal vasculature. This pattern of tenascin-C staining identifies a subdomain in the ontogeny of vessel development between the more distal regions as identified by factor VIII staining and more proximal regions which are positive for tenascin-C but negative for collagen type IV, Laminin and fibronectin EDA domain. This subdomain may represent a prematuration phase prior to the formation of a more stable vasculature marked by pronounced tenascin-C staining. Potentially, tenascin-C may support stable association of smooth muscle cells or pericytes with the developing vasculature, however, in several reports tenascin-C expression has been associated with endothelial sprouting and activation suggesting that tenascin-C may also be modulating active remodeling of the primitive capillary bed as well as stabilization of pericyte association.

Using collagen type IV as a marker for vessel formation the pattern of integrin expression was examined. Data analysis from frozen sections indicated that β_3 integrin was principally expressed on platelets within the developing vasculature. The staining on platelets and not endothelial cells was confirmed by comparing β_3 staining from the corneal burn model with β_3 staining induced by bFGF in the

corneal micropocket assay. Based on these analysis the $\alpha_v\beta_3$ integrin does not appear to play a functional role in endothelial cell mediated migration and angiogenesis within this model system. Further support for this conclusion is the recent report by Klotz *et al.*, in which LM609, an $\alpha_v\beta_3$ specific inhibitory antibody, failed to inhibit angiogenesis within this model system, although a modest but statistically significant inhibition was seen by LM609 in bFGF induced angiogenesis in the rat cornea. The presence of a β_3 specific band in the RT-PCR analysis may represent the expression of $\alpha_v\beta_3$ on macrophages which are present in high abundance throughout the time period studied. Alternatively, the β_3 mRNA message detected by RT-PCR maybe the result of expression in endothelial cells, which showed a low level of staining localized to the lumenal surface. This may reflect a response of endothelial cells within this model similar to that observed in response to ischemic insult in which high levels of VEGF are also present. Functionally this may facilitate platelet or leukocyte adhesion within the developing neovasculature.

Within the developing neovasculature α_1, α_2 and α_5 integrins expression was seen to co-localize with collagen type IV in association with vessel formation at 72 hrs. At later time points (120-168 hrs) α_1 integrin was uniformly expressed within the developing neovasculature, while α_2 appeared to be more prevalent in regions of vessel maturation. The α_5 integrin showed a preferential localization to the more distal regions of vessel formation suggesting a role for $\alpha_5\beta_1$ integrin in the invasive and early maturation and remodeling phases of vessel development within this model system. The role of α_1 and α_2 during vessel formation and maturation maybe associated with regulation of MMP activity and increase in collagen synthesis as a new basement membrane is formed. Both α_1 and α_2 have also been shown to be essential for VEGF mediated angiogenesis and suggested to be expressed early in

the angiogenic in response to VEGF. This also appears to be the case within this model system, however, in later phases of the angiogenic response only α_1 was consistently detected in the more distal regions of vessel formation associated with bud formation and endothelial cell invasion.

β_5 integrin staining was seen throughout the developing vasculature during the early and late phases of vessel formation, however, β_5 integrin appeared more prevalent within distal regions of the developing vasculature. These data suggest that in this model system the $\alpha_v\beta_5$ integrin is associated with vessel development and not $\alpha_v\beta_3$. The association of α_1, α_2, and β_5 integrins in the angiogenic response in the corneal alkaline burn is in keeping with VEGF mediated angiogenic events [12] and the previously observed up regulation of VEGF expression associated with corneal angiogenesis. However, the presence of α_5 integrin within the nascent vasculature also suggests that α_5 β_1 may also play a significant role, potentially in mediating endothelial cell invasion and tubule formation. Involvement of $\alpha_5\beta_1$ in both endothelial cell migration and tubule formation has been demonstrated in in vitro model systems. Although, functional analysis in a VEGF driven pathway has failed to demonstrate an essential role for $\alpha_5\beta_1$.

The other aspect of angiogenesis studied was the expression and activation of MMPs. Within this study the activities of three MMPs were examined. This included MMP-9, MMP-2 and MT1-MMP. Activities of MMP-9 and MMP-2 were addressed by gelatinase zymography and in situ zymography while that of MT1-MMP was inferred by the presence of active MMP-2 and positive immunostaining for MT1-MMP. Both MMP-2 and MT1-MMP were found to be present within this model system and based upon both zymographic and immunohistochemical analysis shown to be associated with the angiogenic response. The correlation

between MMP-2 activation and MT1-MMP immunoreactivity suggests that MT1-MMP is associated with the activation of MMP-2 in this model system. While the data suggest that MT1-MMP is involved in MMP-2 activation other mechanisms of MMP-2 may also be present. Currently, MMP-2 and MT1-MMP are believed to form a functional complex in conjunction with $\alpha_v\beta_3$ and TIMP-2 on the cell surface which in turn mediates localized pericellular proteolysis of the ECM facilitating direction migration and invasion of endothelial cells. Inhibition of this complex formation has been shown to inhibit an angiogenic response further establishing the functional importance of MT1-MMP and MMP-2 in mediating an angiogenic response. However, in the alkaline induced corneal angiogenesis model $\alpha_v\beta_3$ does not appear to play a major role in mediating the angiogenic response and thus the role of MT1-MMP and MMP-2 within this models may function outside of their association with $\alpha_v\beta_3$. Recently, MT1-MMP has been shown to directly mediate cell migration and adhesion through modulation of integrin activity independent of MMP-2. Potentially within the current model system, where $\alpha_v\beta_3$ is not present, MT1-MMP may be directly regulating endothelial cell activity by modulating either $\alpha_v\beta_5$ or beta 1 integrins that co-distribute with MT1-MMP in neovessels.

In addition to MMP-2 and MT1-MMP we also observed increased levels of MMP-9 for both the pro and activated forms. Both the temporal and spatial pattern of MMP-9 expression and activity suggested its association with wound healing and migration of corneal epithelial cells. This, however, does not eliminate a potential role of MMP-9 in regulating the angiogenic response through the generation of angiostatins or release of pro-angiogenic factors from the matrix. Whether MMP-9 plays either a pro-angiogenic or anti-angiogenic role in this model system remains to be determined. Potential activities associated with release of pro angiogenic factors maybe associated with the early degradation of tenascin-C in the scaleral

spur which is observed within the initial 24 hrs after wounding. This response appears to be specific to the angiogenic response since simple corneal debriment does not result in degradation of tenascin-C within the scaleral spur.

In conclusion, the $\alpha_v \beta_5$ integrin appears to be the principal α_v integrin associated with endothelial cells within the corneal alkaline burn model of inflammatory mediated angiogenesis. In addition to $\alpha_v \beta_5$, the $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_5 \beta_1$ integrin showed consistent localization to the developing vasculature bed. Of particular significance was the preferential localization of $\alpha_5\beta_1$ to more distal regions of the developing vasculature. Examination of tenascin–C staining suggests that tenascin-C expression is associated with vessel maturation and has allowed the identification of a novel domain between the invasive front and putative vessel maturation which is tenascin-c negative but collagen type IV positive. Finally, within this model both MT1-MMP and MMP-2 appear to be involved in mediating the angiogenic response although their activity appears to be outside of the formation of a functional complex with $\alpha_v\beta_3$.